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(54) Title: BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA

(57) Abstract: The present invention pertains to the use of microorganisms belonging to the genus *Bifidobacterium* for preparing a carrier for the treatment or prophylaxis of diarrhea. The invention also relates to food or pharmaceutical compositions containing such microorganisms.



**WO 01/10453 A2**



1/10/02

**Bifidobacteria capable of preventing diarrhea**

- 5 The present invention pertains to the use of non-pathogenic microorganisms of the genus Bifidobacterium for preparing a carrier for the treatment or prophylaxis of diarrhea brought about by rotaviruses, and to food or pharmaceutical compositions containing such microorganisms.
- 10 Organisms that produce lactic acid as a major metabolic component have been known for a long time. These bacteria may be found in milk or in milk processing factories, respectively, living or decaying plants but also in the intestine of man and animals. These microorganisms, summarized under the term "lactic acid bacteria", represent a rather inhomogeneous group and comprise e.g. the genera Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium,
- 15 Pediococcus etc..

Lactic acid bacteria have been utilized as fermenting agents for the preservations of food taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. In addition, lactic acid

20 bacteria have also been used for preparing from milk a variety of different foodstuff such as cheese, yogurt and other fermented dairy products.

Quite recently, lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to man and animals upon ingestion. In

25 particular, specific strains of Lactobacillus or Bifidobacterium have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the well-being of man and animal.

In this respect, EP 0 768 375 discloses specific strains of the genus Bifidobacterium, that are

30 capable to become implanted in the intestinal flora and may adhere to intestinal cells. These

Bifidobacteria are reported to assist in immunomodulation, being capable to competitively exclude adhesion of pathogenic bacteria to intestinal cells, thus assisting in the maintenance of the individual's health.

5 During the last few years research has also focused on the potential use of lactic acid bacteria as probiotic agents. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural microflora in the intestine. A microbial preparation may be commonly accepted as a probiotic in case the effectual microbes thereof and their mode of action are known. Probiotics are deemed to attach to the  
10 intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

15 In this respect, WO 97/00078 discloses a specific strain, termed Lactobacillus GG (ATCC 53103), as such a probiotic. The microorganism is particularly employed in a method of preventing or treating food induced hypersensitivity reactions in that it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment with  
20 pepsin and/or trypsin. The Lactobacillus strain selected is described as exhibiting adhesive and colonizing properties and showing a protease enzyme system, so that the protein material contained in the foodstuff to be administered is further hydrolysed by means of proteases secreted by the specific Lactobacillus strain. The method discussed in this document shall eventually result in the uptake of protein material by the gut that does not show a substantial  
25 amount of allergenic material anymore.

Further, in EP 0 577 903 reference is made to the use of such lactic acid bacteria having the ability of replacing *Helicobacter pylori*, the acknowledged cause for the development of ulcer, in the preparation of a support intended for the therapeutic or prophylactic treatment of an  
30 ulcer associated with the action of *Helicobacter pylori*.

In view of the valuable properties particular strains of lactic acid bacteria may exhibit there is a desire in the art to find additional properties of bacterial strains beneficial to the well being of man and/or animal.

- 5 Consequently, the problem underlying the present invention is to provide additional lactic acid bacteria that may exert beneficial activities to living beings upon ingestion.

In the course of the studies leading to the invention it was now surprisingly found that microorganisms of the genus *Bifidobacterium* show properties not yet recognized in the art.

- 10 In effect, the present invention provides for the use of microorganisms belonging to the genus *Bifidobacterium* and being capable to essentially prevent infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.

- The *Bifidobacteria* to be used are preferably selected from the group consisting of  
15 *Bifidobacterium adolescentis* or *Bifidobacterium longum*, preferably *Bifidobacterium adolescentis*, and is more preferably *Bifidobacterium* CNCM I-2168.

- The microorganisms may be used for the preparation of a variety of ingestable carriers, such as e.g. milk, yogurt, curd, fermented milks, milk based fermented products, fermented cereal  
20 based products, milk based powders, infant formulae or pet food and may be included in the respective carrier in an amount of from about  $10^5$  cfu / g to about  $10^{11}$  cfu / g. For the purpose of the present invention the abbreviation cfu shall designate a "colony forming unit" that is defined as number of bacterial cells as revealed by microbiological counts on agar plates.

- 25 The present invention also provides for a food or a pharmaceutical composition containing at least one of the *Bifidobacterium* strains capable to essentially prevent infection of intestinal cells by rotaviruses.

- For preparing a food composition according to the present invention at least one of the  
30 *Bifidobacterium* strains used according to the present invention is incorporated in a suitable

support, in an amount of from about  $10^5$  cfu / g to about  $10^{11}$  cfu / g, preferably from about  $10^6$  cfu / g to about  $10^{10}$  cfu / g, more preferably from about  $10^7$  cfu / g to about  $10^9$  cfu / g.

In case of a pharmaceutical preparation the product may be prepared in form of tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding or a wet tube feeding etc., with the amount of Bifidobacterium strains to be incorporated therein being in the range of up to  $10^{12}$  cfu / g, preferably from about  $10^7$  cfu / g to about  $10^{11}$  cfu / g, more preferably from about  $10^7$  cfu / g to about  $10^{10}$  cfu / g.

- 10 The microorganisms may further be formulated in the carrier so as to obtain a desired release pattern, such as encapsulation etc. Based upon the desired objective the person skilled in the art will select the appropriate excipients and/or additives.

- 15 The activity of the microorganisms in the individual's intestine is of course dose dependent. That is, the more the microorganisms are incorporated by means of ingesting the above food material or the pharmaceutical composition, respectively, the higher the protective and/or curing activity thereof. Since the used microorganisms are not detrimental to mankind and animals and have eventually been isolated from a natural surrounding, namely baby feces, a high amount thereof may be incorporated so that essentially a high proportion of the individual's intestine will be colonized by the microorganisms.

In the figures,

- 25 Fig. 1 shows a scheme illustrating the cell culture screening for assessing rotaviral protective properties of bacterial strains.

- 30 During the extensive studies leading to the present invention the inventors have investigated different bacterial strains isolated from baby feces or obtained from the American Tissue and Cell Collection (ATCC 15704). The different strains were examined for their capability to prevent infection of intestinal cells with rotaviruses that are known to cause diarrhea.

Several bacterial genera comprising Bifidobacterium, Lactococcus, Streptococcus were screened for their rotavirus inhibitory properties. The tests for the inhibitory property were essentially performed with three rotavirus serotypes representing the major etiological agents of human viral diarrhea (serotypes G1, G3 and G4).

5

The various lactic acid bacteria were grown in a suitable medium, such as MRS, Hugo-Jago or M17 medium at temperatures of from about 30 to 40°C corresponding to their optimal growth temperature. After reaching stationary growth the bacteria were collected by centrifugation and re-suspended in physiological NaCl solution. Between the different tests the bacterial cells were stored frozen (-20°C).

10

The various rotavirus stocks were prepared by infection of confluent cell monolayers. The rotaviruses were incubated before infection. The cells were infected with 20 tissue culture infectious doses.

15

For assessing anti-rotaviral properties two different protocols were applied. According to one protocol the various bacterial strains were examined for their direct interaction with the rotavirus strain while in the second protocol the bacteria were screened for those strains that interact with cellular rotavirus receptors.

20

The first protocol involved contacting the respective bacterial suspension each with a different rotavirus strain and incubating in suitable media. Subsequently, the virus-bacteria mixture was applied to a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 (intestinal epithelial cell line) and incubation was continued. Virus replication was then assayed.

25

The second protocol involved incubating the respective bacterial suspension first together with a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 and adding the virus subsequently. After continued incubation virus replication was assayed.

30

Rotavirus replication may easily be assessed by histo-immunological staining of rotavirus proteins in infected cells.

5 A rotavirus inhibitory effect was attributed to a given bacterium when the number of infected cells was reduced by 90% in the cell culture inoculated with rotavirus plus the indicated bacteria in comparison with cells inoculated only with rotavirus.

10 Out of a total of 260 different bacterial strains primarily isolated merely 4 could be shown to essentially inhibit rotaviral replication. The different bacteria were ascertained to belong to the genus *Bifidobacterium* subspecies *adolescentis* or *longum*. One strain belonging to the species *Bifidobacterium adolescentis*, which has been termed Bad4, has been deposited in accordance with the Budapest Treaty and has received the deposit number CNCM I-2168. This strain proved to be extremely effective in preventing infection of human cells by rotaviruses.

15 The present invention will now be described by way of example.

Media and solutions:

MRS (Difco),

20 Hugo-Jago (Tryptone Difco 30 g/l, Yeast Extract Difco 10 g/l, Lactose Difco 5 g/l,  $\text{KH}_2\text{PO}_4$  5 g/l, Beef Extract Difco 2 g/l, agar Difco 2 g/l)

M17 (Difco)

M199 (Seromed)

Ringer solution (Oxoid)

25 PBS ( $\text{NaCl}$  8g/l,  $\text{KCl}$  0.2 g/l,  $\text{Na}_2\text{HPO}_4$  1.15 g/l,  $\text{KH}_2\text{PO}_4$  0.2 g/l))

Tryptose phosphate broth (Flow)

Trypsin-EDTA solution (Seromed)

30 Human rotavirus Wa (G1 serotype) and simian rotavirus SA-11 (G3 serotype) were obtained from P.A. Offit, Children's Hospital of Philadelphia, U.S.A. The DS-1xRRV reassortant



virus was obtained from A. Kapikian, NIH Bethesda, U.S.A. The serotypé 4 human rotavirus Hocht was obtained from P. Bachmann, University of Munich, Germany.

#### Example 1

##### 5 Isolation of lactic acid bacteria from baby feces

Fresh feces were harvested from diapers of 16 healthy babies 15 to 27 days old. 1 g of fresh feces was placed under anaerobic conditions for transportation to the laboratory and microbiological analyses were run within 2 hours from sampling by serial dilutions in Ringer solution and plating on selective media. MRS agar plus antibiotics (phosphomycin 80  $\mu$ g/ml, sulfamethoxazole 93  $\mu$ g/ml, trimethoprim 5  $\mu$ g/ml) incubated at 37°C for 48 hours was used to isolate lactic acid bacteria. Colonies were randomly picked up and purified. Physiological and genetic characterisation was performed on the isolates. In the tests another strain obtained from ATCC (ATCC 15704) was also used, which corresponds to the preferred strain Bad4 to be used according to the present invention.

#### Example 2

##### Testing of strains in cell culture for anti-rotaviral activity

Several bacterial genera comprising Bifidobacterium, Lactococcus and Streptococcus were selected and tested for members which showed anti-rotaviral activity in the cell culture inhibition test (see below 1<sup>st</sup> and 2<sup>nd</sup> protocol). The genus Lactococcus was represented by a single species (Lc. lactis) consisting of two subspecies (Lc. lactis subsp. lactis and cremoris). A total of 30 strains were tested. The Streptococcus genus was represented by a single species (S. thermophilus) with 45 strains. The Leuconostoc and Propionibacterium genus were only represented by a single species (6 strains), while the Enterococcus and Staphylococcus genus was represented by two species each and a total of 17 strains.

In total, 260 bacterial strains were tested for rotavirus inhibitory activity.

1<sup>st</sup> protocol:

30 µl of the respective bacterial suspension (containing on average  $3 \times 10^6$  bacteria) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) to which were added 100 µl of virus in supplemented M199 medium. The virus-bacteria mixture thus obtained was incubated for 1 hour at 4°C and for 1 hour at 37°C. Separately, cells of the human undifferentiated colon adenoma cells HT-29 growing as a confluent monolayer in 96-well microtiter plates (in M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) 1 : 4 diluted with PBS) were washed three times with phosphate-buffered saline (PBS ; pH 7.2). The virus-bacteria mixture processed as indicated above was transferred to the cells and the microtiter plates were incubated for 18 h in a CO<sub>2</sub> incubator (Heraeus). Virus replication was assayed as described below.

2<sup>nd</sup> protocol:

30 µl of the bacterial suspension (supra) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed ) and applied directly on HT-29 cells grown and pretreated as described in the 1<sup>st</sup> protocol in the microtiter plates. After one hour incubation at 37°C 100 µl of virus in supplemented M199 medium were added to the cells in the microtiter plates. The incubation was continued for 18 h in a CO<sub>2</sub> incubator (Heraeus). Virus replication was assayed as described below.

The rotavirus replication was assessed by histo-immunological staining of rotavirus proteins in infected cells as described hereafter.

One day after infection, the cell culture medium was removed from the microtiter plates and the cells were fixed with absolute ethanol for 10 min. Ethanol was discarded, and the plates were washed three times in PBS buffer. Then 50 µl of an anti-rotavirus serum (mainly directed against VP6 protein), produced in rabbits (obtained from the ISREC University of Lausanne) and diluted 1 :2000 in PBS was added to each well and incubated for 1 h at 37°C with a cover slip to prevent desiccation of the wells. The anti-serum was discarded afterwards

and the plates were washed three times with PBS. Then 50  $\mu$ l of anti-rabbit immunoglobulin G (IgG) antiserum produced in goats and coupled to peroxidase (GAR-IgG-PO; Nordic) were added at a dilution of 1 : 500 in PBS to each well and the plates were incubated for 1 hour at 37 °C. The serum was discarded and the plates were again washed three times with  
5 PBS. Then 100  $\mu$ l of the following substrate mixture was added to each well : 10 ml of 0.05 M Tris-hydrochloride (pH 7.8), 1 ml of H<sub>2</sub>O<sub>2</sub> (30% suprapur, diluted 1 :600 in H<sub>2</sub>O ; Merck) and 200  $\mu$ l of 3-amino-9-ethylcarbazole (0.1 g/10 ml of ethanol stored in 200  $\mu$ l aliquots at – 80 °C ; A-5754 ; Sigma). The plates were incubated for at least 30 min at room temperature. The substrate was discarded and the wells were filled with 200  $\mu$ l of H<sub>2</sub>O to stop the reaction.  
10 Infected cell foci were counted with an inverted microscope (Diavert ; Leitz).

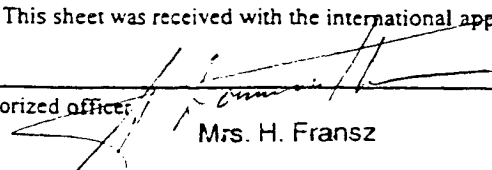
Only very few bacterial strains interacted with rotaviruses. Merely 4 out of the 260 bacterial cells primarily selected inhibited rotavirus replication in at least one protocol. Bifido-  
bacterium adolescentis CNCM I-2168 (Bad4) showed an extremely high activity against  
15 Serotype 1 Rotavirus, Serotype 3 rotavirus SA-11 and Serotype 4 rotavirus Hocht.

Bad4 is gram positive and catalase negative, it does not produce CO<sub>2</sub> during fermentation and produces just L (+) lactic acid according to methods disclosed in the "Genera of lactic acid bacteria", Ed. B.J.B. Wood and W.H. Holzapfel, Blackie A&P.

**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>3</u> . line <u>16</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float:right">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Collection Nationale de Cultures de Microorganismes Institut Pasteur	
Address of depositary institution (including postal code and country) 25, Rue du Docteur Roux F-75724 Paris Cedex 15	
Date of deposit 15/03/1999	Accession Number NCC 251 - I-2168
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float:right">This information is continued on an additional sheet <input type="checkbox"/></span>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

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## Claims

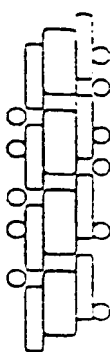
1. Use of a lactic acid bacterium belonging to the genus *Bifidobacterium* capable of preventing infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.
2. The use according to claim 1, wherein the *Bifidobacterium* is selected from the group consisting of *Bifidobacterium longum* or *Bifidobacterium adolescentis*.
3. The use according to claim 1, wherein the *Bifidobacterium* is *Bifidobacterium* CNCM I-2168.
4. The use according to any of the preceding claims, wherein the *Bifidobacterium* is contained in an ingestible carrier.
5. The use according to claim 5, wherein the *Bifidobacterium* is contained in the carrier in an amount from about  $10^5$  cfu / g to about  $10^{12}$  cfu / g carrier.
6. The use according to any of the claims 4 or 5, wherein the carrier is a food composition selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae or pet food.
7. Food or pharmaceutical composition containing at least one *Bifidobacterium* strain capable of preventing infection of intestinal cells by rotaviruses.
8. The composition according to claim 7, which is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, pet food, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement, dry tube feeding or wet tube feeding.

9. The composition according to claim 7, which is in form of a tablet, liquid bacterial suspension, dried oral supplement, wet oral supplement, dry tube feeding or a wet tube feeding.

# Cell Culture Screening

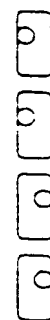


Culture protected



Protective:  
SerotypeG1: Bad4  
SerotypeG3: Bad4  
SerotypeG4: Bad4

Culture infected



Non-Protective:  
n=250 LAB strains

Human intestinal  
cell line HT-29



FIG. 1





# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

# PCT

<p>To:</p> <p>STRAUS, Alexander BECKER-KURIG-STAUS Bavariastrasse 7 D-80336 München ALLEMAGNE</p>	<div style="border: 1px solid black; padding: 5px; margin: 0 auto; width: 80%;"> <p><b>BECKER-KURIG-STAUS</b></p> <p><b>15. Nov. 2001</b></p> <p>WV: ..... / LF: .....</p> </div>
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NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT  
(PCT Rule 71.1)

<p>Date of mailing (day/month/year) 15.11.2001</p>		<p><b>IMPORTANT NOTIFICATION</b></p>	
<p>Applicant's or agent's file reference 80188/WO</p>			
<p>international application No. PCT/EP00/07207</p>	<p>international filing date (day/month/year) 26/07/2000</p>	<p>Priority date (day/month/year) 05/08/1999</p>	
<p>Applicant SOCIETE DES PRODUITS NESTLE S.A. et al.</p>			



1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

#### 4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

<p>Name and mailing address of the IPEA/</p> <div style="text-align: center;">  </div> <p>European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465</p>	<p>Authorized officer</p> <p>Exner, K</p> <p>Tel. +49 89 2399-7826</p> <div style="text-align: right;">  </div>
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2000 20 NOV 2001

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference 80188/WO	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/07207	International filing date (day/month/year) 26/07/2000	Priority date (day/month/year) 05/08/1999
International Patent Classification (IPC) or national classification and IPC A61K35/74		
Applicant SOCIETE DES PRODUITS NESTLE S.A. et al.		
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 5 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>		
<p>3. This report contains indications relating to the following items.</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input type="checkbox"/> Priority</p> <p>III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p>IV <input type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input type="checkbox"/> Certain documents cited</p> <p>VII <input type="checkbox"/> Certain defects in the international application</p> <p>VIII <input checked="" type="checkbox"/> Certain observations on the international application</p>		
Date of submission of the demand  26/01/2001	Date of completion of this report  15.11.2001	
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Smetankine, L  Telephone No. +49 89 2399 8466	





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/07207

**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-9 as originally filed

**Claims. No.:**

1-9 as originally filed

**Drawings, sheets:**

1/1 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/07207

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes:	Claims	3
	No:	Claims	1,2,4-9
Inventive step (IS)	Yes:	Claims	3
	No:	Claims	1,2,4-9
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations  
**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:  
**see separate sheet**





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/EP00/07207

**POINT VIII:**

Claim 5 should refer to claim 4 ( and not to claim 5 ).

**POINT V:**

1. Novelty and inventive step:

EP - A - 904 784 (1) - see column 2 lines 34,47, column 8 line 29, describes the use of lactic acid bacterium belonging to the genus Bifidobacterium such as B. adolescentis and B. longum ( see column 2 line 50 ) for the treatment of diarrhea. Further column 8 lines 27 to 33 (0059) specify that diarrhoea is caused by pathogens such as rotaviruses.

Carrier consisting in food composition containing milk, yogurts, fermented milk, milk based fermented products, liquid bacterial suspension, dried oral supplement, wet oral supplement, are also described by (1) - see column 3 lines 29- 33, column 4 lines 47 to 54, thus claims 1,2 and 4 to 9 seem to lack novelty.

US - A - 5 902 578 (2) - see column 1 lines 5 to 13, describes the use, subject - matter of claim 1, wherein the Bifidobacterium is contained in an ingestible carrier such as milk or a nutritional supplement, with an amount of  $1.75$  to  $8.75 \times 10^6$  CFU/g or  $6-7 \times 10^{10}$  CFU ( column 4 lines 32-33 and column 5 line 43, claim 4 ) - the carrier being a dried oral supplement ( column 2 lines 38-42 ), milk, fermented milk ( column 2 lines 42 to 50 ), wet oral supplement ( column 4 lines 29-35 ), thus the prevention or the treatment of diarrhea associated with rotavirus is therefore disclosed, thus claims 1,4 to 9 seem to be not new.

Further, ANAEROBE 1997, vol. 3, n°2-3, pp.73-78 (3) - see page 74 right - hand column , paragraph 2, describes Bifidobacterium longum as commercially available yogurts.

Therefore with respect with the above cited documents, documents (1) and (2) described the use of Bifidobacterium strains for the prevention of infections of intestinal cells by rotaviruses, thus this document resolved the problem solved by the present application by the same solution, thus if the objections concerning the lack of novelty would be met, these claims 1,2 4 to 9 would lack inventive step.



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/EP00/07207

The use of the specific bacterium, subject - matter of claim 3 seems to be new. The extremely high activity of this bacterium against rotavirus ( page 9 second paragraph of the present application ) could not be deduced from the prior art, thus claim 3 seem to be inventive.



INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 3, line 16.

## B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet: ☐

Name of depositary institution

Collection Nationale de Cultures de Microorganismes  
Institut Pasteur

Address of depositary institution (including postal code and country)

25, Rue du Docteur Roux  
F-75724 Paris Cedex 15

Date of deposit

15/03/1999

Accession Number

NCC 251 - I-2168

## C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet: ☐

## D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

## E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only

☐ This sheet was received with the international application

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer



# CNCM

Paris, le 15 mars 1999

Collection Nationale  
de Cultures de Microorganismes

**INSTITUT PASTEUR** EINBOGANGEN

25, Rue du Docteur Roux  
F-75724 PARIS CEDEX 15

22. März 1999

Tel : (33-1) 45 68 82 55  
Fax : (33-1) 45 68 82 36

Monsieur Roman VUILLE  
SOCIETE DES PRODUITS NESTLE S.A.  
Patents Department  
Avenue Nestlé 55  
CH-1800 VEVEY  
SUISSE

Envoi par télécopie aux numéros :

(41) 21 924 28 80 / (41) 21 785 89 25

N/R : CNCM-6742.903

Obj : l'enregistrement de quatre bactéries en vue de dépôts aux termes du Traité de Budapest

Cop : Madame Aline MAMIN, Monsieur Roberto RENIERO

Nestlé Research Centre (CRN), Vers-chez-les-Blanc, C.P. 44, CH-1000 LAUSANNE 26

Monsieur,

Par la présente nous vous confirmons avoir reçu ce jour, en vue de quatre dépôts initiaux suivant la règle 6.1 du Traité de Budapest, douze lyophilisats relatifs à chacun des microorganismes identifiés ci-dessous.

Vos projets de dépôt ont été enregistrés à la CNCM  
à la date du **15 mars 1999** sous les numéros suivants

Référence d'identification

Numéro d'enregistrement CNCM

NCC 251

I-2168

NCC 481

I-2169

NCC 490

I-2170

NCC 585

I-2171

Si un dépôt est accepté, le numéro d'ordre attribué par la CNCM est identique au numéro d'enregistrement et la date du dépôt est la date de l'enregistrement.

Restant à votre disposition,

nous vous prions d'agréer, Monsieur, l'expression de notre considération distinguée.

Mme Y. CERISIER  
Directeur Administratif de la CNCM





From the INTERNATIONAL SEARCHING AUTHORITY

**PCT**

To:

BECKER-KURIG-STRAUS  
Attn. STRAUS, Alexander  
Bavariastrasse 7  
D-80336 München  
GERMANY

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference

80188/W0

Date of mailing  
(day/month/year)

30/01/2001

**FOR FURTHER ACTION**

See paragraphs 1 and 4 below

International application No.

PCT/EP 00/07207

International filing date

(day/month/year)

26/07/2000

Applicant

SOCIETE DES PRODUITS NESTLE S.A.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

**For more detailed instructions,** see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2  
NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Catherine Humbert



These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

## INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

### What parts of the International application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the International application is to be published.

### What documents must/may accompany the amendments?

**Letter (Section 205(b)):**

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.



The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

**"Statement under article 19(1)" (Rule 46.4)**

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

**Consequence if a demand for international preliminary examination has already been filed**

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

**Consequence with regard to translation of the international application for entry into the national phase**

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.



## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>80188/WO</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 00/ 07207</b>	International filing date (day/month/year) <b>26/07/2000</b>	(Earliest) Priority Date (day/month/year) <b>05/08/1999</b>
Applicant <b>SOCIETE DES PRODUITS NESTLE S.A.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.  
☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.





## INTERNATIONAL SEARCH REPORT

International Application No

T/EP 00/07207

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/74 A23C9/00 C12N1/20 A61P1/12 //(C12N1/20,  
C12R1:04)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, EMBASE, LIFESCIENCES, SCISEARCH, FSTA, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 904 784 A (NUTRICIA) 31 March 1999 (1999-03-31) the whole document ---	1,4-9
X	US 5 902 578 A (HALPIN-DOHNALEK M. I. ET AL.) 11 May 1999 (1999-05-11) the whole document ---	1,4-9
X	MC FARLAND L V ET AL: "Pharmaceutical probiotics for the treatment of anaerobic and other infections." ANAEROBE 1997, vol. 3, no. 2-3, 1997, pages 73-78, XP002137052 ISSN: 1075-9964 the whole document --- -/--	1,4-9

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*G\* document member of the same patent family

Date of the actual completion of the international search

16 January 2001

Date of mailing of the international search report

30/01/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J



## INTERNATIONAL SEARCH REPORT

International Application No

T/EP 00/07207

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 768 375 A (SOCIÉTÉ DES PRODUITS NESLÉ) 16 April 1997 (1997-04-16) cited in the application the whole document ---	1-9
X	DUFFY LINDA C: "Interactions mediating bacterial translocation in the immature intestine." JOURNAL OF NUTRITION., vol. 130, no. 2 suppl., February 2000 (2000-02), pages 432S-436S, XP000978813 ISSN: 0022-3166 the whole document -----	1-9



## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

T/EP 00/07207

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 904784	A	31-03-1999	NONE	
US 5902578	A	11-05-1999	WO 9735596 A	02-10-1997
EP 768375	A	16-04-1997	EP 0577904 A	12-01-1994
			AT 172245 T	15-10-1998
			DE 69227329 D	19-11-1998
			DE 69227329 T	01-04-1999
			DK 768375 T	23-06-1999
			ES 2124060 T	16-01-1999
			AT 153063 T	15-05-1997
			AU 673525 B	14-11-1996
			AU 4158793 A	13-01-1994
			CA 2099856 A	07-01-1994
			CZ 9301343 A	16-02-1994
			DE 69219768 D	19-06-1997
			DK 577904 T	27-10-1997
			ES 2102485 T	01-08-1997
			FI 933002 A	07-01-1994
			GR 3024219 T	31-10-1997
			HK 1000143 A	24-12-1997
			HU 68567 A	28-06-1995
			IE 80629 B	21-10-1998
			JP 2916350 B	05-07-1999
			JP 6315373 A	15-11-1994
			NO 932408 A	07-01-1994
			NZ 248057 A	28-08-1995
			PL 299542 A	21-02-1994
			RO 115175 B	30-11-1999
			RU 2126446 C	20-02-1999
			SK 280682 B	12-06-2000
			SK 71293 A	06-07-1994
			US 5494664 A	27-02-1996
			US 5603930 A	18-02-1997

